SPECIFICATION

METHOD OF PRODUCING TRANSGENIC PLANTS HAVING IMPROVED AMINO ACID COMPOSITION AND IMPROVED YIELDING

Background of the Invention

The present invention relates to transgenic plants having an increased free amino acid content, and a method of producing them. In particular, the present invention relates to transgenic plants containing at least one of asparagine, aspartic acid, serine, threonine, alanine, histidine and glutamic acid accumulated in a large amount, and a method of producing them and also the present invention relates to a method of increasing an yield of potato, a transgenic potato of which yielding may be increased and a method of producing the transgenic potato.

The technique of transforming a plant by introducing a specified gene was firstly reported in the world in the study where it was achieve by introducing a gene into tobacco with *Agrobacterium tumefaciens*, a soil microorganism. Thereafter, many products having useful agricultural features were produced, and it was also tried to let plants produce useful components. A plant breeding method by such a transgenic plants producing technique is considered to be hopeful in place of the ordinary, traditional breeding technique, such as cross-fertilization. Among them, improvement in the characteristics of plants concerning nitrogen assimilation is also being studied. Particularly, the study of amino acids which are encompassed in the products is particularly prospering because they are important ingredients in fruits, tubers, roots of root crops and seeds and also they exert a great influence on the tastes.

Reports on the biosynthesis of amino acids include, for example, a report that free lysine content of tobacco was increased to 200 times as high

content by introducing *E. coli* dihydrodipicolinate synthase DHDPS gene into tobacco (U. S. Patent No. 5,258,300, Molecular Genetics Res. & Development); a report that free lysine content was increased by introducing aspartate kinase AK gene (EP 485970, WO 9319190); a report that asparagine content was increased to 100 times as high content by introducing asparagine synthetase AS gene into tobacco (WO 9509911, Univ. New York, WO 9013533, Univ. Rockfeller); and a report that tryptophan content was increased to 90 times as high content by introducing an anthranilic acid-synthesizing enzyme into a rice plant (WO 9726366, DEKALB·Genetic Corp). The plants into which a gene is incorporated are not limited to model plants such as tobacco and *Arabidopsis thaliana* but plants which produce fruits such as tomato are also used. For example, as for tomatoes, a transformant thereof was obtained by *Agrobacterium* co-cultivation method in 1986 [S. McCormick, et.al., Plant Cell Reports, 5, 81-84 (1986); Chyi, et. al., Mol. Gen. Genet., 204, 64-69 (1986)]. Since then, investigations were made for the improvement of the transforming system. Various genes relating to the biosynthesis of amino acids and nitrogen assimilation other than those described above are also known. They include asparaginase and glutamine synthase (GOGAT), and the nucleotide sequences of them were also reported.

Glutamic acid which is a kind of α-amino acids is widely distributed in proteins and is used for seasonings. It is known that a tasty component of tomato and also a tasty component of fermentation products of soybeans (such as soy sauce and fermented soy paste) are all glutamic acid. It is also known that glutamic acid is synthesized in the first step of nitrogen metabolism in higher plants. It is also known that glutamine and asparagine generated from glutamic acid are distributed to tissues through phloems and used for the synthesis of other amino acids and proteins. It was reported that in plants, photosynthesis products such as sucrose and amino acids are present in a high concentration in the fluid of phloems which are the transporting pathway of the products [Mitsuo Chino et al., "Shokubutsu Eiyo / Hiryogaku" p. 125 (1993)].

As the example where the photosynthesis products are contained in a high concentration in edible parts of plants, it is known that about 0.25 g/100 g f. w. of the photosynthesis products is contained in tomato fruits ["Tokimeki" No. 2, Nippon Shokuhin Kogyo Gakkaishi, Vol. 39, pp. 64-67 (1992)]. However, it can not be easily conducted to accumulate glutamic acid in a high concentration in plant bodies, because it is a starting material for amino group-donors and also it is metabolized in various biosynthetic pathways as described above even though the biosynthesizing capacity of the source organs may be improved. As far as the applicant knows, it has never been succeeded to remarkably increase glutamic acid concentration in plants by either mated breeding or gene manipulation.

The first step of the assimilation of inorganic nitrogen into an organic substance is mainly the incorporation of ammonia into glutamic acid to generate glutamine, which is catalyzed by glutamine synthase (GS). Then glutamine, with α-ketoglutaric acid, are catalyzed by glutamate synthase (GOGAT) and generate 2 molecules of glutamic acid. This GS/GOGAT cycle is considered to be the main pathway of nitrogen assimilation in plants [Miflin and Lea, Phytochemistry 15; 873-885 (1976)]. On the other hand, it is also known that the ammonia assimilation proceeds also through a metabolic pathway other than the pathway wherein ammonia is incorporated by being catalyzed by GS [Knight and Langston-Unkefer, Science, 241: 951-954 (1988)]. Namely, ammonia is incorporated into α-ketoglutaric acid to form glutamic acid, which is catalyzed by glutamate dehydrogenase (GDH). However, plant GDH has a high Km value for ammonia. The role of this pathway under normal growing conditions has not yet been elucidated enough because ammonia is toxic and the concentration of intracellular ammonia is usually low. It is reported in an investigation that the ammonia contributes to the nitrogen assimilation when ammonium concentration in the cells is increased over a normal level (Knight and Langston-Unkefer, supra.).

In plants, glutamate dehydrogenase (GDH) catalyzes the reversible

reaction in which ammonia is released from glutamic acid to generate α-ketoglutaric acid, adversely, ammonia is incorporated into α-ketoglutaric acid to generate glutamic acid as discussed above. It is considered that the former occurs when the content of ammonia nitrogen is high, and the latter occurs when nitrate nitrogen content is high [Robinson et al., Plant Physiol. 95; 809-816 (1991): and Robinson et al., Plant Physiol. 98; 1190-1195 (1992)]. The directionality of this enzyme is not clear, unlike GDH-A enzyme which acts in microorganisms to synthesize glutamic acid or GDH-B enzyme which acts on them to decompose it. In plants, it is considered that there are two kinds of such enzymes, i.e. NADP-depending GDH which functions in chloroplasts and NAD-dependent GDH which functions in mitochondrias. Since GDH has a high Km value for ammonia and it is highly related to the ammonia level during the photorespiration, it is supposed that NAD-dependent GDH localized in mitochondria has an important role in the assimilation of ammonia [Srivastava and Singh RP, Phytochemistry, 26; 597-610 (1987).

It is known that plant GDH comprises a hexamer composed of two different kinds of polypeptides (α -subunits and β -subunits) linked with each other at random and that there are seven isozyme patterns depending on the degree of the linkage. After investigations wherein grapevine calli were used, the following facts were reported: When calli cultured in a medium containing a nitrate and glutamic acid were subjected to electrophoresis, an isozyme comprising β-subunits was increased on the cathodic side. On the contrary, when calli cultured in a medium containing ammonia and glutamine were subjected to the electrophoresis, an isozyme comprising α-subunits was increased on the anodic side. Further, when the calli were transferred from the nitrate medium into the ammonia medium, GDH activity was increased 3-fold as high activity (α-subunits were increased 4-fold and β-subunits were decreased), the activity was moved from the cathod region to the anode region [Loulakakis and Poubelakis – Angelakis, Plant Physiol. 97; 104-1111 (1996)]. According to this report, α-subunits were considered to play an important role in the

assimilation of ammonia.

In 1995, Sakakibara et al. [Plant Cell Physiol., 33; 1193-1198] isolated GDH gene of a plant for the first time from two isozyme bands on the cathodic region in seven isozyme bands in maize roots. Thereafter, GDH genes were isolated from grapevine [Syntichaki et al., Gene 168: 87-92 (1996)], *Arabidopsis* [Melo-Olivera et al., Proc. Natl. Acad. Sci., USA 93; 4718-4723 (1996)] and tomato [Purnell et al., Gene 186; 249-254 (1997)]. In particular, the genes isolated from grapevine calli were isolated from an isozyme expressed in ammonia-treated cells, and they are considered to be the genes encoding α-subunits. All the genes contained a transit peptide which is functional in mitochondria. GDH genes of maize or tomato are expressed in a large amount in the root, while those of *Arabidopsis* are expressed in the leaves and flowers. It was reported that only one copy of gene is present in tomato, while two or more genes are present in maize, *Arabidopsis* and grapevine, which. suggested that the constitution and function of genes are different among plants and complicated.

Transgenic plants into which said GDH gene was introduced were also produced. It was reported that when glutamate dehydrogenase GDH (NADP-GDH) gene from *Escherichia coli* was introduced into tobacco and maize for the purpose of imparting resistance to phosphinothricin used as a herbicide, the glutamic acid content of the roots of them was increased to 1.3 to 1.4 times as high [Lightfoot David et al, CA 2180786 (1966)]. According to this report, glutamic acid content of tobacco roots was increased from 14.7 mg/100 gf.w. to 20.6 mg/100 gf. w., and that of maize roots was increased from 16.2 mg/100 gf. w. to 19.1 mg/100 gf. w. Additionally, Lightfoot et al. reported that the glutamic acid content was significantly reduced in seeds of the transformed maize into which GDH gene had been introduced (U. S. Patent No. 5,998,700). Although there are other reports on the use of GDH gene, no example is given therein [WO 9509911, α, β-subunits from chlorella (WO 9712983)]. In addition, no analytical value of amino acids of glutamic acid group was given therein.

Additionally, photosynthetic phosphoenolpyruvate carboxylase (C_4-PEPC) gene and NADP-dependent malate dehydrogenase (cpMDH) gene (Beaujean et al., Plant Science 160:1199-1210 (2001)), GA20-oxidase gene (Carrera et al., Plant J. 22: 247-256 (2000)), Hexokinase 1 gene (Veramendi et al., Plant Physioil 121: 123-134 (1999)), NADP-dependent cytosolic isocitrate dehydrogenase (Kruse et al., Planta 205: 82-91 (1998)), ADP glucose pyrophosphorylase gene (Greene et al., Proc Natl. Acad. Sci. USA 93 (1996): 1509-1513; Rober et al., Planta 199: 528-536 (1996)), or cytosolic fructose-1,6-bisphosphatase gene (Zrenner et al., Plant J. 9: 671-681 (1996)) was introduced into potatoes, in order to modify the starch content in potato tubers, but the yield of potato tubers was not substantially affected. Redros et al. (Planta 209: 153-160 (1999)) reported that the number of tubers was increased when S-adenosylmethionine decarboxylase gene was introduced into potatoes and over expressed, but the size of tubers was small and the total weight of tubers did not differ from the total weight of non-transformants. Additionally, Van Asshce et al. (US Patent NO.5981952) reported that sucrose phosphate synthase (SPS) gene was introduced to a potato and the yield of the potato tubers was investigated under various concentration of CO_2, which resulted in the increase of dry weight of a tuber. However, the amount of increase was very small. As far as the applicant knows, it has never been succeeded to remarkably increase the yield of potato tubers.

Summary of the Invention

The object of the present invention is to provide a method of increasing free amino acid contents of storage organs of plants, in particular, at least one of glutamic acid, asparagine, aspartic acid, serine, threonine, alanine and histidine contained in edible parts including roots, tubers, fruits and seeds of plants; and also to provide transgenic plants in which free amino acids are accumulated in a large amount.

Another object of the present invention is to provide a method of

increasing the yield of potato and to provide a transgenic potato of which yield is increased.

The object of the present invention is attained by providing a plant having a changed expression levels and/or expression balance of organ-specific expression of major enzymes concerning the assimilation and utilization of nitrogen, and providing a method of producing such a plant. Such plants can be produced by introducing at least one gene encoding an enzyme which assimilates or utilizes nitrogen together with a suitable regulatory sequence and excessively expressing the same or repressing the expression.

The transgenic plant in which a free amino acid is accumulated in a large amount according to the present invention, particularly a plant in which at least one of glutamic acid, asparagine, aspartic acid, serine, threonine, alanine and histidine, particularly glutamic acid, is accumulated in a large amount, may be obtained by introducing a glutamate dehydrogenase (GDH) gene derived from eucaryote together with a suitable regulatory sequence into the plant and overexpress the gene.

The potato being increased in the yield according to the present invention may be also obtained by introducing a glutamate dehydrogenase (GDH) gene derived from eucaryote together with a suitable regulatory sequence to the plant and overexpress the gene.

Brief Description of the Drawings

Fig. 1 shows the comparison of the nucleotide sequence of glutamate dehydrogenase (GDH) *AN-gdh-17* gene from *Aspergillus nidulans* with the known nucleotide sequence of NADP-GDH gene. The upper line shows the nucleotide sequence of NADP-GDH gene, and the lower line shows that of *AN-gdh-17* gene.

Fig. 2 shows the comparison of the nucleotide sequence of glutamate dehydrogenase (GDH) *AN-gdh-17* gene from *Aspergillus nidulans* with the known nucleotide sequence of NADP-GDH gene (continued from Fig. 1). The

upper line shows the nucleotide sequence of NADP-GDH gene, and the lower line shows that of *AN-gdh-17* gene.

Fig. 3 shows the cloning strategy of *AN-gdh-17* gene into Ti plasmid (pMAT037). In the figures, 35S Pro represents CaMV 35S promoter, and Term represents a terminator.

Fig. 4 is a schematic view showing the strategy of constructing a genetic construct containing the sequence encoding *AN-gdh-17* gene from *Aspergillus* connected to the transit peptide, i. e. pCt-AN-gdh, pCt-dAN-gdh and pMt-dAN-gdh, and the structures of them.

Fig. 5 is a schematic diagram of the strategy for removing the splicing region from *AN-gdh-17* gene, wherein thick lines show the splicing region, and P1 through P4 each represent a PCR primer.

Fig. 6 is a schematic diagram showing the strategy for connecting the transit peptide sequence to *Aspergillus AN-gdh-17* gene sequence, wherein A represents the nucleotide sequence of the transit peptide, B represents the nucleotide sequence of *AN-gdh-17* gene, and P5 through P8 each represent a PCR primer.

Fig. 7 is a schematic diagram showing the structures of genetic constructs p2ACt-dAN-gdh and p2AMt-dAN-gdh containing *AN-gdh-17* gene from *Aspergillus nidulans* connected to 2A11 promoter.

Fig. 8 shows the cloning strategy of tomato NAD-GDH gene (*T-gdh-4*) into Ti plasmid (pIG121-Hm). In the figure, 35S represents CaMV 35S promoter, and Nos represents a Nos-terminator.

Fig. 9 is a schematic diagram showing the strategy of modifying *T-gdh-4*, a tomato NAD-GDH gene.

Fig. 10 shows the result of PCR analysis of transformants into which *Aspergillus nidulans AN-gdh-17* gene was introduced, wherein:

lane 1:λ-HindIII marker;

lanes 2 and 3: untransformed tomatoes (Cont-1 and Cont-2);

lanes 4 to 6: transformed tomatoes obtained by introducing plasmid

(pMAT037) gene;

lanes 7 to 10: transformed tomatoes (No. 6, No. 8-2, No. 15 and No. 17) obtained by introducing *AN-gdh-17* gene; and

lane 11: 100bp marker.

Fig. 11 shows a result of PCR analysis of transformants obtained by introducing GDH (*T-gdh-4*) gene which is tomato NAD-GDH gene.

lane 1:λ-HindIII marker;

lanes 2 and 3: untransformed tomatoes;

lanes 4 to 6: transformed tomatoes obtained by introducing plasmid (pIG121-Hm) gene;

lanes 7 to 10: transformed tomatoes (No. 2, No. 7-2, No. 9-2 and No. 10) obtained by introducing *T-gdh-4* gene; and

lane 11: 100bp marker.

Fig. 12 shows a result of RT-PCR analysis of a transformant into which *Aspergillus nidulans AN-gdh-4* gene was introduced,

lane 1: 100bp marker;

lane 2: untransformed tomatoes;

lanes 3 and 5: transformed tomato No.6;

lane 4: transformed tomato No.15.

The tissues from which the total RNA was extracted were shown in the parenthesis.

Fig. 13 shows a result of RT-PCR analysis of a transformant into which tomato *T-gdh-4* gene was introduced, wherein Nos. 2, 7-2, 9-2 and 10 each represent a transformed tomato, and tissues from which the total RNA was extracted were shown in the parenthesis.

Fig. 14 is a graph showing the comparison of amino acid (glutamic acid - Glu, glutamine - Gln, γ-aminobutyric acid-GABA and lysine - Lys) content of the transformants (No.6, No.15 and No.17) into which *AN-gdh-17* gene was introduced.

Fig. 15 shows a result of the comparison of amino acid (glutamic acid -

Glu, glutamine - Gln, γ-aminobutyric acid-GABA and lysine -Lys) content of the transformants (No.2, No.7-2, No.9-2, No.10) into which *T-gdh-4* gene was introduced.

Fig. 16 shows the result of Southern analysis of transgenic tomatoes (T_1) into which the *AN-gdh-17* gene was introduced. The samples used were prepared by digesting the total DNA (15 μg) with *Bam* HI and *Eco*RI (A) or with *Xba* I (B).

lane 1: untransformed tomato;

lane 2: AN-gdh-17 No.1;

lane 3: AN-gdh-17 No.3;

lane 4: AN-gdh-17 No.15;

lane 5: AN-gdh-17 No.2.1.

Fig. 17 shows the result of Southern analysis of transgenic tomato (T1) into which *T-gdh-4* gene was introduced. The samples used were prepared by digesting the total DNA (15 μg) with *Xba* I and *Sac* I.

lane 1: untransformed tomato;

lane 2: T-gdh No.1-2;

lane 3: T-gdh No.3-1;

lane 4: T-gdh No.8-1.

The arrow indicates the location of the band corresponding to the introduced gene (1.2kb)

Fig. 18 shows amino acids contents in fruits of T_1 tomato into which *AN-gdh-17* gene had been introduced. An untransformed tomato was used as a control. Each measurement was conducted using 3 plants.

Fig. 19 shows amino acids contents in fruits of T_1 tomato into which *T-gdh-4* gene was introduced. An untransformed tomato was used as a control. Each measurement was conducted using 3 plants.

Fig. 20 shows a Southern analysis of transgenic potatoes into which *Ct-AN-gdh* or *Mt-dAN-gdh* gene was introduced. *Eco* RI digest of the total DNA (15μg) was used.

lane 1: untransformed potato-1;

lane 2: untransformed potato-2;

lanes 3-6: corresponding to a transformed potato into which each genetic construct was introduced; lane 3:Ct-AN-gdh No.1, lane 4: Mt-dAN-gdh No.2, lane 5: Mt-dAN-gdh No.5, lane 6:Mt-dAN-gdh No.8.

The arrow indicates the location of the band corresponding to the introduced gene segment (1.5 kb)

Fig. 21 is a graph showing the contents of Glu in microtubers of potato transformed with *Ct-AN-gdh* or *Mt-dAN-gdh* gene. An untransformed potato was used as a control.

Fig. 22 is a graph showing the fresh weight of the above ground part of the potato into which *Mt-dAN-gdh* gene was introduced.

Fig. 23 is a graph showing the fresh weight of tubers of the potato into which *Mt-dAN-gdh* gene was introduced.

Description of the Preferred Embodiments

The present invention relates to a genetic manipulation of the nitrogen metabolism in plant. Particularly, the present invention relates to modifying the expression level of the enzymes involved in the nitrogen assimilation or nitrogen utilization to increase free amino acids, particularly glutamic acid which is the tasty ingredient, in edible parts of useful plants such as fruits, tubers, roots of root crops or seeds. These enzymes are enhanced in their expression or modified, or inhibited to produce the pants having the desired properties.

The target genes used in the present invention are those encoding enzymes concerning the assimilation of ammonia to amino acids. An example of the target genes is glutamate dehydrogenase (GDH). The expression of this enzyme is enhanced and, in addition, it is modified (for example, ectopic expression by addition of a transit sequence) to produce a plant having desired properties. Particularly, the yielding of potato may be increased by enhancing or modifying the expression (for example, ectopic expression by the addition of a

transit sequence) of GDH in potato.

The manipulation may be carried out by transforming a plant with a nucleic acid construct described herein. The transformed plants or their descendants express desired, modified enzymes and they are screened for attaining a change in expression of corresponding mRNA, change in the assimilation or utilization of nitrogen and/or increase in free amino acid content of plants.

In short, the method of the present invention comprises the following steps, and the transgenic plants of the present invention are those produced by this method:

a) the step of cloning the intended gene;

b) the step of recloning the obtained gene into a suitable vector, if necessary;

c) the step of introducing the vector into plant cells to obtain a transformant; and

d) the step of regenerating the obtained transformant to a plant and cultivating it.

In one of the embodiments of the present invention, one or several genes encoding the enzyme(s) for assimilating or utilizing nitrogen are placed under the control of a strong constitutive promoter, and over-expressed in the plant bodies. The modification of the expression may be accomplished by the gene manipulation of the plants by utilizing at least one of the followings:

a) a transgene in which a gene sequence encoding an enzyme is operably connected to a strong constituting promoter,

b) native multi copy genes, which encodes a desired enzyme,

c) a regulatory gene for activating the expression of an intended gene for assimilating or utilizing nitrogen.

d) a native single copy gene, which is modified in order to increase the expression and which has a regulatory site, and

e) a transgene which expresses a variant, modified or chimera enzyme for

assimilating or utilizing nitrogen.

In another embodiment of the present invention, the expression pattern of the enzyme for assimilating or utilizing nitrogen is modified. The expression pattern may be modified by the gene manipulation of the plants by utilizing at least one of the followings:

a) a transgene in which a gene sequence encoding an enzyme is operably connected to a promoter having a desired expression pattern (such as a promoter having an organ-specific or development stage-specific expression pattern).

b) a modification regulatory gene which activates the expression in a preferred pattern of a gene encoding the enzyme, and

c) a native single copy gene, which has a regulatory site, modified so as to express in a preferred pattern.

In still another embodiment of the present invention, a modified enzyme or enzyme of a different type is represented in a pathway of assimilating or utilizing nitrogen. This type of embodiment involves the production of a genetic construct which can be expressed in plant cells and which encodes a corresponding enzyme having a catalytic effect different from a catalytic effect of the enzyme, which assimilates or utilizes nitrogen in a host plant and also the gene manipulation with the genetic construct. By such procedures, plants containing free amino acids in an increased amount may be obtained.

For breeding such plants, a conventional method of breeding new varieties of plants is unsuitable because it requires screening of large isolated groups and a long time. However, by employing the methods of the present invention, such a labor becomes unnecessary and the time can be saved.

The terms and abbreviations used herein are defined as follows:

CaMV35S: cauliflower mosaic virus 35S promoter

NADP-GDH: NADP-dependent glutamate dehydrogenase

NAD-GDH: NAD-dependent glutamate dehydrogenase

Fused gene construct: a genetic construct comprising a promoter in which different genes are connected together (the promoter controls the transcription of heterologous genes)

Heterologous gene: In a genetic construct, a heterologous gene means a gene which is connected with a promoter which is not naturally linked to the gene. The heterologous gene may be from the organism which provide the promoter.

GABA: γ-aminobutyric acid.

The genes of the enzymes usable in the present invention may be derived from, but not limited to, bacteria, yeasts, alga, animals and plants. They can be obtained also from various other sources. The sequences obtained from those sources may be connected to a suitable promoter which functions in plant cells in such a manner that the function is not disturbed. The *in vitro* mutagenesis or *de novo* synthesis is also possible in order to enhance the translation efficiency in the host plants or to change the catalytic effect of the encoded enzyme. The modification includes the modification of the residue concerning catalytic functions but is not limited thereto. The transgene can be modified so as to have an optimum codon depending on the codon usage of the host or the organelle to be expressed. If necessary, such a gene sequence may be connected to a nucleic acid sequence encoding a suitable transit peptide.

A preferred modification also includes a construction of hybrid enzymes. For example, different domains of related enzymes obtained from the same or different organisms may be combined with each other to generate an enzyme having a new property.

In addition, nucleic acid segments capable of hybridizing with the

above described various nucleic acid sequences under stringent conditions can also be used in the present invention so far as the desired activity is not lost. Thus, nucleic acid segments encoding a protein, in which one or more amino acids are deleted, added or replaced, are also included. The term "stringent conditions" indicates ordinary conditions well known by those skilled in the art such as described by Sambrook et al [as described above (1989)]. Nucleic acid sequences capable of hybridizing under such conditions will usually have at least 60 %, preferably at least 80 % and particularly preferably at least 90 % of homology to each other.

Various genes are included in the enzyme genes for assimilating or utilizing nitrogen usable in the present invention. Glutamate dehydrogenase (GDH) gene is one of examples of preferred enzymes usable for accumulating glutamic acid. When GDH gene is used, it is expressed in the sense direction. When GDH gene is selected, it is preferably expressed as a fusion gene having a transit peptide at the 5' region. Particularly preferred transit peptides are a transit peptide for mitochondria and that for chloroplast.

A preferred embodiment of the present invention will be illustrated with reference to an example wherein a tomato plant or a potato plant is manipulated by a genetic engineering technique with a recombinant genetic construct encoding NADP-dependant GDH gene from a fungus (*Aspergillus nidulans*) [Alastair et al., Mol. Gen. Genetics, 218, 105-111 (1989)] or tomato NAD-dependant GDH gene [Purnell et al., Gene 186; 249-254 (1997)] functionally connected to the cauliflower mosaic virus (CaMV) 35S promoter which is a powerful constitutive plant promoter. In the lines where GDH is excessively expressed, free amino acid content in the edible parts is increased as compared with that of the control untransformed plant. In particular, glutamic acid content is increased to 2- to 3-fold.

The nucleic acid constructs usable in the present invention can be prepared by the methods well known by those skilled in the art. For example, the recombinant DNA techniques which can be used for isolating the

components of a construct, determining their features, handling them, and generating the construct, can be found in, for example, Sambrook et al., Molecular cloning-Laboratory manual, the second edition (Cold Spring Harbor Laboratory Press). When a nucleotide sequence of a desired component is known, it is advantageous not to isolate it from a biological source but to synthesize it. In such a case, those skilled in the art may refer to literatures such as Caruthers et al., Nuc. Acids. Res. Symp. Ser. 7: 215-233 (1980) and Chow and Kempe, Nuc. Acids. Res. 9: 2807-2817 (1981). In other cases, the desired component may be advantageously produced by polymerase chain reaction (PCR) amplification. As for PCR method, those skilled in the art can refer to Gelfand, "PCR Technique (The Theory and Application of DNA Amplification)" edited by H. A. Erlich and published by Stockton Press, N. Y. in 1989 and "Present Protocol in Molecular Biology" Vol. 2, Chapter 15 edited by Ausubel et al., and published by John Wiley & Sons in 1988.

The genetic constructs used in the present invention may generally contain a suitable promoter which functions in plant cells, a suitable terminator such as nopaline synthetic enzyme gene terminator, other elements useful for regulating the expression and marker genes suitable for selecting the transformant such as drug-resistant genes, e. g. genes resistant to kanamycin, G 418 or hygromycin in addition to the intended gene. The promoter contained in the genetic construct may be a constitutive promoter, an organ-specific promoter or a developmental stage-specific promoter and can be suitably selected depending on the host, gene, desired expression level, organ for the expression, developmental stage, etc.

According to the present invention, a plant showing an overexpression of an enzyme for assimilating or utilizing nitrogen can be obtained by transforming plant cells with a genetic construct containing a plant promoter connected to a sequence encoding a desired enzyme. In a preferred embodiment of the present invention, related promoters are powerful, organ-unspecific or developmental stage-unspecific promoters (such as promoters

which strongly express in many or all tissues). An example of such a powerful, constitutive promoters is CaMV35S promoter.

In another embodiment of the present invention, it is advantageous in some cases that a plant is manipulated with a genetic construct in which an organ-specific or growing stage-specific promoter is linked with a sequence encoding a desired enzyme. For example, when the expression in a photosynthetic tissues and organs is intended, a promoter of ribulose bisphosphate carboxylase (RuBisCO) gene or chloroplast a/b binding protein (CAB) gene is usable. When the expression in seeds is intended, promoters of various seed storage protein genes are usable. When the expression in fruits is intended, a fruit-specific promoter (such as tomato 2A11) is usable. When the expression in tubers is intended, a promoter of protein genes stored in tubers (such as potato patatin) is usable.

In still another embodiment of the present invention, it may be advantageous to transform a plant with a genetic construct in which an inducible promoter connected to a sequence encoding the desired enzyme. Examples of such promoters are diversified, which include, but are not limited to: heat shock genes, protection responding genes (such as phenylalanine ammonia lyase gene), wound responding genes (such as cell wall protein genes rich in hydroxyproline), chemically inducible genes (such as nitrate reductase gene and chitinase gene) and dark inducible genes (such as asparagine synthetase gene (Coruzzi and Tsai, U. S. Patent No. 5,256,558).

The recombinant nucleic acid genetic construct of the present invention may contain a selectable marker for tracing the transmission of the genetic construct. For example, a genetic construct transmitted in bacteria preferably contains an antibiotic-resistant gene such as kanamycin resistant, tetracycline resistant, streptomycin resistant or chloramphenicol resistant genes. The suitable vectors to transfer the genetic construct include plasmids, cosmids, bacteriophages and viruses. In addition, the recombinant genetic construct may contain a selectable marker gene or a marker gene which can be

screened, which can be expressed in plants, for isolating, identifying or tracing the plant cells transformed with the genetic construct. The selective markers include, but are not limited to, genes which impart resistance to an antibiotic (such as kanamycin or hygromycin) or resistance to a herbicide (such as sulfonylurea, phosphinothricin or glyphosate). The markers which may be screened include, but are not limited to, genes encoding β-glucuronidase [Jefferson, Plant Mol. Biol. Rep 5: 387-405 (1987)], genes encoding luciferase [Ow et al., Science 234: 856-859 (1986)] and B and CI gene products controlling the production of anthocyanin pigment (Goff et al., EMBO J, 9: 2517-2522 (1990)).

The methods of gene introducing which may be employed in the present invention are not particularly limited. Any method known in the art for transferring a gene into plant cells or plant bodies may be employed. For example, in an embodiment of the present invention, *Agrobacterium* may be used for introducing a genetic construct into a plant. In such a transformation, it is desirable to use binary *Agrobacterium* T-DNA vector [Bevan, Nuc. acid Res. 12: 8711-8721 (1984)] and co-culture [Horsch et al, Science, 227: 1229-1231 (1985)]. *Agrobacterium* transformed system is usually used for manipulating dicotyledons [Bevans et al., Ann. Rev. Genet., 16: 357-384 (1982); and Rogers et al., Methods Enzymol., 118: 627-641 (1986)]. *Agrobacterium* transformed system is also usable for transforming monocotyledons and plant cells [Hernalsteen et al., EMBO J., 3: 3039-3041 (1984); Hoykass-Van Slogteren et al., Nature, 311: 763-764 (l984); Grimsley et al., Nature, 325: 167-1679 (1987); Boulton et al., Plant Mol. Biol., 12: 31-40 (1989); and Gould et al., Plant Physiol., 95: 426-434 (1991)]. When the *Agrobacterium* system is used for the transformation of plants, the recombinant DNA genetic construct further contains at least right border sequence of T-DNA region at a position adjacent to DNA sequence to be introduced into plant cells. In a preferred embodiment, the sequence to be introduced is inserted between the left and right T-DNA border sequences. Suitable designs and constructions of transformed vectors

based on T-DNA are well known in the art.

In another embodiment, various other methods may be employed for introducing the recombinant nucleic acid genetic construct into plants or plant cells. An example of other gene introduction method and transformation method is a protoplast transformation of naked DNA by calcium, polyethylene glycol (PEG) or electroporation [Paszkowski et al., EMBO J., 3: 2717-2722 (1984); Potrykus et al., Mol. Gen. Genet., 199: 169-177 (1985); Fromm et al., Proc. Nat. Acad. Sci. USA, 82: 5824-5828 (1985); and Shimamoto et al., Nature, 338: 274-276 (1989)]. According to the present invention, various plants and plant cells can be manipulated to obtain desired physiological properties described herein by using the nucleic acid construct and the transformation method, as described above. The methods of the present invention are particularly advantageous when the target product is a monocotyledon or plant cells. In a preferred embodiment, the target plants and plant cells to be manipulated include, but are not limited to, tomato, potato, beet, soybean, *Arabidopsis*, maize, wheat, rice plant and sugar cane.

According to the present invention, an intended plant can be obtained by introducing and manipulating a genetic construct as disclosed herein into various plant cells including, but are not limited to, protoplasts, tissue-cultured cells, tissues and organ explants, pollens, embryos and whole plant bodies. From the plants manipulated according to the embodiment of the present invention, the intended transgenic plant is selected or screened by an approach and method described below. An individual plant body may be regenerated from the isolated transformant. Methods of regenerating individual plant bodies from plant cells, tissues or organs for various species are well known by those skilled in the art.

The transformed plant cells, calli, tissues or plants may be identified and isolated by selecting or screening the characters encoded by marker genes contained in the genetic construct used for the transformation. For example, the selection may be conducted by growing a manipulated plant in a medium

containing a repressive amount of antibiotic or herbicide, to which the introduced genetic construct can impart the resistance. Further, the transformed plant cells and plants may be identified by the screening with reference to the activity of visible marker genes (such as β-glucuronidase genes, luciferase genes, B genes or CI genes) which may be present in the transgenic nucleic acid construct of the present invention. The methods of the selection and screening are well known by those skilled in the art.

Physical methods and biochemical methods can be employed for identifying plants containing the genetic construct of the present invention or plant cells transformed with the construct. Examples of the methods include:

1) Southern analysis or PCR amplification for detecting and determining the structure of recombinant DNA insert;

2) Northern blotting, S1 RNase protection, primer elongation PCR amplification or reverse transcriptase PCR (RT-PCR) amplification for detecting and determining the RNA transcription product of genetic construct; and

3) When the genetic construct is a protein, protein gel electrophoresis, western blotting, immune precipitation, enzyme immunoassay, etc. but the methods are not limited to them. These assay methods are well known by those skilled in the art.

According to the present invention, the transformed plant can be screened for an intended physiological change for the purpose of obtaining the plant having improved component characters. For example, when the manipulation is conducted for overexpression of GDH enzyme, the transformed plant can be tested for the expression of GDH enzyme at a desired level in a desired tissue or in a desired growing stage. Then the plant having a desired physiological change, such as overexpression of GDH gene, may be successively screened with reference to a desired change in the components.

According to the present invention, plants manipulated by modifications in the process for assimilating or utilizing nitrogen have improved component characteristics. Namely, they may contain a large amount of free

amino acids, particularly glutamic acid, asparagine, aspartic acid, serine, threonine, alanine and histidine, especially they may contain glutamic acid in a particularly large amount, which is a tasty component. The manipulated plants and plant lines having such improved characters can be identified by determining free amino acid contents of the plants. The operation and method of the analysis are well known by those skilled in the art. Additionally, the yield of potato can be increased according to the present method. The potato having this improved property may be identified by simply cultivating potatoes and determining the number of tubers or the total weight of the tubers and the like.

The plants obtained by the present invention have free amino acid contents higher than those of control plants (untransformed plants). Untransformed plant as used herein is a plant that is not yet transformed with a genetic construct which is capable of expressing GDH. In a preferred embodiment, free amino acid content, particularly glutamic acid (tasty component) content in edible parts such as fruits, tubers, roots and seeds of a desired plant is increased to at least twice as high as that of the parent. The total amino acid content is also increased to 2 to 4 times as high as that of the parent. As for amino acids other than glutamic acid, the increase in amount of particularly aspartic acid, asparagine, alanine, serine, threonine and histidine is remarkable.

The potatoes which may be obtained according to the present invention, are the plant having increased yield compared to a control plant (untransformed plant). Untransformed plant is a plant which is not yet transformed with a genetic construct which is capable of expressing GDH. In a preferred embodiment, the total weight (g) of the tuber part increases in statically significant amount, generally at least 1.5-fold, preferably at least 2-fold, and the number of tubers also increases.

Examples

The present invention will be concretely illustrated in detail by the

following Examples relating to the production of engineered plants for overexpression of NADP-GDH gene or NAD-GDH gene.

Example 1 Isolation of GDH gene from *Aspergillus nidulans* and tomato, and construction of Ti plasmid

(1) Isolation of NADP-dependent GDH gene (*AN-gdh-17*) from *Aspergillus nidulans* and NAD-dependent GDH gene (*T-gdh-4*) from tomato

A. nidulans was plated and cultured on potato dextrose agar medium at 30°C overnight. The colonies thus obtained were further cultured in a dextrose liquid medium for 2 days. Total RNA was produced from the propagated microbes.

Tomato seeds surface-sterilized with 70 % ethanol (30 seconds) and 2 % sodium hypochlorite (15 minutes) were placed on plant hormone-free MS agar medium [Murashige and Skoog, Physiol. Plant., 15: 473-479 (1962)], and cultured at 25°C for one week while the daylight hours were kept to be 16 hours to obtain sterile plants. Total RNA was prepared from the roots of the obtained seedlings.

As for the total RNA, mRNA was purified with Poly (A) Quick mRNA Isolation Kit (Stratagene Co.) and then First-Strand cDNA was produced with First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biothech Co.). The PCR reaction was conducted with First-Strand cDNA, thus obtained, as a template. The PCR reaction was conducted with PCR system 2400 (Perkin Elmer) as follows: 35 cycles under conditions of 94°C-3 minutes, 94°C-45 seconds, 59°C-30 seconds, 72°C-90 seconds; and then 72°C-10 minutes. The primers used are shown in Table 1. As a result, a band of about 1.4 kbp from *A. nidulans* and that of about 1.2 kb from tomato were amplified and they were coincident with the expected sizes of the intended genes. Obtained PCR products were cloned with TA-cloning kit (Invitrogen Co.).

The sequences of 2 clones of plasmids into which the genes of an intended size from *A. nidulans* were cloned and also the sequences of 5 clones

of plasmids into which the genes of an intended size from a tomato root were determined with a sequencer (377A of ABI Co.), and the homology of them to known NADP-dependent GDH gene from *A. nidulans* [Alastair et al., Mol. Gen. Genet. 218; 105-111 (1989)] and GDH gene from tomato [Purnell et al., Gene 186; 249-254 (1997)] was examined.

The nucleotide sequence of one of two clones derived from *A. nidulans* (*AN-gdh-17*) coincided with known nucleotide sequence of NADP-GDH gene (Fig. 1-2). However, it was found that in two splicing sites, one splicing site of about 50 bp remained in the gene. Because *A. nidulans* has eucaryote-type splicing recognition site, the experiment in the subsequent step was conducted with the remaining splicing site. On the other hand, nucleotide sequences of 2 clones (*T-gdh-4* and *T-gdh-22*) in 5 clones derived form a tomato root coincided with a known *legdh 1* sequence. The nucleotide sequence of *AN-gdh-17* is shown in SEQ ID NO:1, and that of *T-gdh-4* is shown in SEQ ID NO:2.

Table 1 Primer DNA used for PCR reaction

a	5' -region -TCT AGA ATG TCT AAC CTT CCC GTT GAG C- 3' -region (28 mer) (SEQ ID:3)	
	5' -region -GAG CTC TCA CCA CCA GTC ACC CTG GTC C- 3' -region (28 mer) (SEQ ID:4)	
b.	5' -region -TCT AGA ATG AAT GCT TTA GCA GCA ACT- 3' -region (27 mer) (SEQ ID:5)	
	5' -region -GAG CTC TTA CGC CTC CCA TCC TCG AAG- 3' -region (27 mer) (SEQ ID:6)	

a. NADP-GDH gene specific primer [Alastair et al. Mol. Gen. Genet., 218; 105-111 (1989), PCR product, about 1.4 kbp]

b. *legdh*1 specific primer [Purnell et al., Gene 186; 249-254 (1997), PCR product, about 1.2 kbp]

<Sequence Listing Free Text>

SEQ ID NOs:3,4: NADP-GDH specific PCR primer

SEQ ID NOs:5,6: *legdh*1 specific PCR primer

(2) Subcloning of *AN-gdh-17* gene into Ti-plasmid (pMAT037)

AN-gdh-17 gene cloned into PCR2.1 vector was subcloned into Ti plasmid (pMAT037) which was a plant transformation vector [Matsuoka and Nakamura, Proc. Natl. Acad. Sci. USA, 88: 834-838 (1991)]. Because pMAT037 did not have restriction enzyme sites suitable for the direct insertion, the gene was once ligated into pUC18 (*Xba*I, *Eco*RI site used) to transform *E. coli* JM109. It was then ligated with Ti plasmid through *Pst*I site and *Eco*RI site in pUC18 to obtain plasmid pAN-gdh-17 (Fig. 3 and Table 2). The transformation into *E. coli* DH5 α was conducted. *Agrobacterium* strain EHA101 was transformed with Ti plasmid pAN-gdh-17 (Fig. 3) in which *AN-gdh-17* had been introduced. Obtained *Agrobacterium* pAN-gdh-17/EHA101 was used for the infection of tomatoes.

(3) Construction of pCt-AN-gdh, pCt-dAN-gdh and pMt-dAN-gdh

NADP-GDH gene from *Aspergillus nidurans* has originally two splicing sites. Although these splicing sites should have been removed by the amplification of GDH gene with cDNA by PCR method, *AN-gdh-17* gene obtained in the experiment still had one splicing site of about 50 bp remaining therein (Fig. 1, see nucleotide sequence). Therefore, the remaining nucleotide sequence of about 50 bp was removed by PCR method (Table2, Figs. 4-5).

The strategy for the construction of these genetic constructs is shown in Fig. 5. In this strategy, DNA segments were amplified by PCR method by using primer P1 containing 5' terminal of the cloned gene sequence, primer 2 containing 5' side and 3' side of the splicing site but free of the splicing site, primer P3 containing 5' side and 3' side of the splicing site but free of the splicing site, and primer P4 containing 3' terminal of *AN-gdh-17* gene and also using *AN-gdh-17* gene as the template. Then the size of the PCR product was

confirmed by the electrophoresis and the product was re-extracted from the gel. The re-extracted PCR products were mixed together. PCR reaction was conducted again by using primers P1 and P4. The obtained PCR products were cloned and sequenced to confirm that the splicing site was accurately removed.

The sequences of the above-described P1 to P4 were as follows:

P1; 5'-TCTAGAATGTCTAACCTTCCCGTTGAGC-3' (SEQ ID:7)

P2; 5'-CACCCATGTTTAGTCCTGTGAGAG-3' (SEQ ID:8)

P3; 5'-CTCTCACAGGACTAAACATGGGTG-3' (SEQ ID:9)

P4; 5'-GAGCTCTCACCACCAGTCACCCTGGTCC-3'(SEQ ID:10)

Thus, *dAN-gdh-17* gene from which the splicing site had been removed was obtained (Table 2, Figs. 4-5).

To make the action of the introduced gene more functional at a suitable position, the transit peptide sequence for mitochondria or chloroplast was connected to the upstream of the initiation codon of *AN-gdh-17* gene or *dAN-gdh-17* gene (Table 2, Fig. 4). As for the peptide sequences used, a nucleotide sequence (about 70 bp) connected to tomato GDH gene was used as the transit peptide sequence for mitochondria, and a nucleotide sequence (about 120 bp) connected to the small subunit gene of tomato RuBisCO was used as the transit peptide sequence for chloroplast. These genes were connected to *AN-gdh-17* gene or *dAN-gdh-17* gene by PCR method. The transit peptide sequence for mitochondria was obtained by using two primers(5'-GGATCCATGAATGCTTTAGCAGCAAC-3' : sequence SEQ IDNO:11, and 5'-TCTAGATAAACCAAGAAGCCTAGCTG-3': sequence SEQ IDNO:12) by PCR. The transit peptide sequence for chloroplast was obtained by using two primers (5'-CTGCAGATGGCTTCCTCAATTGTCTCATCG-3' : sequence SEQ ID NO:13, and 5'-TCTAGAGCATCTAACGCGTCCACCATTGCT-3': sequence

SEQ ID NO:14) by PCR .

These transit peptide sequences were linked with *AN-gdh-17* gene or *dAN-gdh-17* gene as shown in Fig. 6. Namely, the respective DNA segments were amplified by using primer P5 corresponding to 5' side of the transit peptide, primer P6 containing the sequence of 3' terminal of the transit peptide and the sequence at 5' terminal of *AN-gdh-17* gene or *dAN-gdh-17* gene, primer P7 containing sequences of 3' terminal of the transit peptide and 5' terminal of *AN-gdh-17* gene or *dAN-gdh-17* gene, and primer P8 containing sequences of 3' terminal of *AN-gdh-17* gene or *dAN-gdh-17* gene. Then the size of the PCR product was confirmed by the electrophoresis. After the extraction from the gel, the extracted segments were mixed together and again subjected to PCR with primers P5 and p8. The amplified segments were cloned and then sequenced to confirm that the nucleotide sequence of the transit peptide was correctly connected to *AN-gdh-17* gene or *dAN-gdh-17* gene (Fig.6).

The sequences of P5 to P8 are as shown below.

The primer used for linking with the transit peptide sequence for mitochondria:

P5; 5'-TCTAGAATGAATGCTTTAGCAGCAAC-3'(SEQ ID:15)

P6; 5'-GGGAAGGTTAGACATTAAACCAAGAAGCCT-3' (SEQ ID:16)

P7; 5'-AGGCTTCTTGGTTTAATGTCTAACCTTCCC-3' (SEQ ID:17)

P8; 5'-GAGCTCTTACGCCTCCCATCCTCGAA-3' (SEQ ID:18)

The primer used for linking with the transit peptide sequence for chloroplast:

P5; 5'-CTGCAGATGGCTTCCTCAATTGTCTCATCG-3' (SEQ ID:19)

P6; 5'-AAGGTTAGACATGCATCTACCGCG-3' (SEQ ID:20)

P7; 5'-CGCGTTAGATGCATGTCTAACCTT-3' (SEQ ID:21)

P8; 5'-GAGCTCTTACGCCTCCCATCCTCGAA-3' (SEQ ID:22)

AN-gdh-17 gene was introduced into the sense direction in the multicloning site of Ti plasmid pMAT037 (Fig. 3). In the construction of pCt-AN-gdh, pCt-dAN-gdh and pMt-dAN-gdh, the cloning was conducted with Ti plasmid pIG121-Hm and the gene was introduced in order to compare the effect obtained by using CaMV35S promoter with that obtained by using fruit-specific promoter gene (2A11) which will be described below (Table 2, Fig. 4).

<Sequence Listing Free Text>

SEQ ID NOs:7-10: PCR primer for removing splicing region

SEQ ID NOs:11,12: PCR primer for amplifying the sequence encoding the mitochondria transit peptide

SEQ ID NOs:13, 14: PCR primer for amplifying the sequence encoding the chloroplast transit peptide

SEQ ID NOs:15-18: PCR primer for producing the sequence encoding the mitochondria transit peptide-GDH

SEQ ID NOs:19-22: PCR primer for producing the sequence encoding the chloroplast transit peptide-GDH

(4) Genetic construction with fruit-specific promoter (2A11 promoter)

Fruit-specific expression promoter (2A11) was obtained by PCR method by using total DNA obtained from tomato seedlings as the template. In the primers (SEQ ID NOs:23 and 24) used, the sequences of restriction enzyme sites, *Hin*dIII and *Xba*I used for the introduction into Ti plasmid were designed respectively.

The sequences of the primers were as follows:

5'-AAGCTTATATAACCCAAAATATACTA-3'(SEQ ID NO:23)

5'-TCTAGAGGTACCATTAATTGCTAATT-3'(SEQ ID NO:24)

After cloning the obtained PCR product with TA cloning kit, the nucleotide sequence thereof was confirmed by the sequence analysis. Obtained 2A11 promoter was replaced with CaMV35S promoter before GUS gene of Ti plasmid pIG121-Hm by using the above-described restriction enzymes, *Hin*dIII and *Xba*I. Then GUS part was replaced with *Ct-dAN-gdh* gene or *Mt-dAN-gdh* gene. The process for the replacement with *Ct-dAN-gdh* gene or *Mt-dAN-gdh* gene was the same as that employed for CaMV35S promoter. From them, plasmids p2Act-dAN-gdh and p2AMt-dAN-gdh were obtained (Fig. 7).

<Sequence Listing Free Text>

SEQ ID NOs:23, 24: PCR primer for amplifying 2A11 promoter sequence

Important structures of plasmids produced as described above are summarized in following Table 2.

Table 2. NADP-GDH construct which was introduced into transgenic tomato.

	Gene body (NADP-GDH)		Transit peptide			Promoter.	
	Splicing region		No	Chl.*1	Mit.*2	35S	2A11
	Contain	Delete					
pAN-gdh-17 (Figure 3)	○		○			○	
pCt-AN-gdh (Figure 4)	○			○		○	
pCt-dAN-gdh (Figure 4)		○		○		○	
pMt-dAN-gdh (Figure 4)		○			○	○	
p2ACt-dAN-gdh (Figure 7)		○		○			○
p2AMt-dAN-gdh (Figure 7)		○			○		○

Chl.*1: Chloroplast, Mit.*2: Mitochondria

(5) Subcloning of *T-gdh-4* gene into Ti plasmid (pIG121-Hm)

Cloned tomato GDH gene (*T-gdh-4*) was introduced into Ti-plasmid (pIG121-Hm) to obtain plasmid pT-gdh-4. In the introduction, *Xba*I site and *Sac*I site previously provided in the primer used in the isolation step were used. Ti plasmid containing *T-gdh-4* gene (Fig. 8) was used to transform *Agrobacterium* strain EHA101.

(6) Subcloning of tomato GDH gene variants into Ti plasmid (pIG121-Hm)

Lysine at position 90 in tomato NAD-GDH which is the glutamic acid binding site was replaced with alanine, and the effect obtained by the

replacement was examined (Fig. 9). This modification was conducted for the purpose of inhibiting the binding with glutamic acid, because GDH gene of higher plants is inclined to reduce the amount of glutamic acid depending on ammonia ion concentration and nutrition conditions. The replacement of one amino acid was conducted by changing AAG encoding Lys to GCG encoding Ala by site-directed mutagenesis using PCR. The altered gene thus obtained was named "*Td-gdh*". Then *Td-gdh* gene sequence was introduced into Ti plasmid to obtain plasmid pTd-gdh (Fig. 9). The strategy is the same as that for *T-gdh-4*.

Example 2. Production of tomato transformants and analysis of them

(1) Production of tomato transformants

Tomato (cultivar, minitomato, Fukukaen Seeds Co.) seeds were surface-sterilized with 70 % ethanol (30 seconds) and 2 % sodium hypochlorite (15 minutes), and then placed on a plant hormone-free MS agar medium. The seeds were cultured at 25°C for one week while the daylight hours were kept to be 16 hours. The cotyledons were taken from the obtained sterile seedlings and then placed on an MS agar medium containing 2 mg/l of Zeatin and 0.1 mg/l of Indoleacetic acid (regeneration medium in 9 cm Petri dish), and cultured under the same conditions as those described above for 2 days. *Agrobacterium* (EHA 101) containing thus constructed gene was cultured in YEP medium (Table 3) overnight and used for the infection. The cotyledons cultured for two days were collected in a sterilized Petri dish, and the *Agrobacterium* suspension was added to them to cause the infection. Superfluous *Agrobacterium* suspension was removed from the cotyledons by using a sterilized filter paper. After further removing superfluous *Agrobacterium* suspension, a sterilized filter paper was then placed on the medium in the above-described Petri dish in order to prevent rapid propagation of *Agrobacterium*. The infected cotyledons were placed thereon and co-cultured for 24 hours.

The cotyledons were transferred to MS regeneration medium (selecting medium) containing 50 mg/l of kanamycin and 500 mg/l of Claforan to select transformants. The regenerated shoots were transferred into a new selecting medium to conduct further selection. Well-grown green shoots were cut at the stems and transferred into a plant hormone-free MS medium (rooting medium in a test tube). The rooted, regenerated plant was acclimated to the soil.

Table 3

Composition of YEP medium	(1 liter)
Bactotrypton	10 g
Yeast extract	10 g
glucose	1 g

(2) Confirmation of the introduced gene

Total DNA was extracted by method of Honda et al. [Honda and Hirai, Jpn. J. Breed 40, 339-348 (1990)] from 4 selected individual plants obtained by the infection with *Agrobacterium* containing *AN-gdh-17 gene*, 4 selected individuals obtained by the infection with *Agrobacterium* containing *T-gdh-4* gene, 3 plant individuals obtained by the infection with *Agrobacterium* (only Ti plasmid) free of the intended gene, and 2 plant individuals obtained from the cotyledons by the direct regeneration without using *Agrobacterium*. DNA thus extracted was purified by RNase treatment, phenol/chloroform treatment and PEG precipitation. The purified product was diluted to 0.01 μg/μl and used as a template for PCR. PCR reaction was conducted with primers 9 and 10 which amplify the region from Nos-Promoter to *NPTII* (PCR products, 1.0 kbp). The reaction conditions were as follows: 35 cycles under conditions of 94°C-1 minute, 55°C-1 minute and 72°C-2 minutes. The PCR product was treated by the electrophoresis with 1 % agarose gel and then stained with ethidium bromide (Figs. 10 and 11).

The primers used were as follows:

P 9 : 5'-CCCCTCGGTATCCAATTAGAG-3' (SEQ ID NO:25)

P 10 : 5'-CGGGGGGTGGGCGAAGAACTCCAG-3' (SEQ ID NO:26)

A band of an intended size (1.0 kb) was observed in four lines infected with *AN-gdh-17* gene and four lines infected with *T-gdh-4 gene* but not in two untransformed lines. From these results, it was confirmed that the gene had been introduced into four lines infected with Ti-plasmid containing *AN-gdh-17* gene and four lines infected with Ti-plasmid containing *T-gdh-4* gene.

<Sequence Listing Free Text>

SEQ ID NO:25, 26: PCR primer for amplifying Nos-Promoter-NTPII region

(3) Confirmation of expression of introduced gene

Then, the expression of the introduced gene was confirmed by RT-PCR by using transformed tomatoes in which the introduction of the intended gene had been confirmed. The total RNA was extracted from leaves and fruits of the lines infected with *Agrobacterium* containing *AN-gdh-17* or *T-gdh-4* gene and in which the introduced genes were confirmed by PCR analysis, and the first strands cDNA were prepared after RNase treatment. Then PCR was conducted with primers (SEQ ID NOs:3 and 4, and SEQ ID NOs:5 and 6), which was used in the isolation of genes, using the first-strand cDNA as the template. The reaction conditions were as follows: 30 cycles under conditions of 94°C-1 minute, 55°C-1 minute and 72°C-2 minutes. As a result, the introduced gene was confirmed to be expressed in all the leaves and fruits (Figs. 12 and 13).

(4) Extraction and quantitative determination of free amino acids

Fruits of the acclimated tomato transformant were harvested 6 weeks after the blossom, and stored at –80°C. Each fruit was cut into about 6 pieces,

weighed, placed in a mortar, frozen with liquid nitrogen and ground. 3 ml of 80 % ethanol was added thereto, and the obtained mixture was further thoroughly ground, transferred into a centrifugal tube and incubated at 80°C for 20 minutes. After the centrifugation at 10,000 rpm for 20 minutes, the supernatant was transferred into another tube. 2 ml of 80 % ethanol was added to the remaining pellets, and the pellets were ground again in the mortar and then incubated at 80°C for 20 minutes. After the centrifugation, the supernatant was transferred into the tube containing the prior supernatant to obtain a mixture. The total amount of the mixture was adjusted to 5 ml with 80 % ethanol. After thorough mixing, 200 μl of the mixture was taken, dried and dissolved in 0.02 N hydrochloric acid. After the filtration through a 0.45 μm filter, the sample for the analysis was obtained. The amino acid analysis was conducted with Hitachi high-speed amino acid analyzer (L-8800).

The amino acid analysis of the fruits (red) of a strain having *AN-gdh-17* gene introduced therein was conducted 6 weeks after the bloom. The results of the analysis are shown in Table 4 together with the results of the analysis of untransformed plants (control plants). In the lines wherein glutamic acid content was remarkably increased, glutamic acid content was increased 1.75-fold (No. 6), 2.54-fold (No. 15) and 2.48-fold (No. 17) (Fig. 14). Amino acids other than glutamic acid, such as asparagine, aspartic acid, alanine, serine, threonine and histidine, were also increased in amount.

Table 4. Amino acid content of transformed tomato (T_0 generation) into which *AN-gdh-17* gene was introduced

	Asp	Thr	Ser	Asn	Glu	Gln	Gly	Ala	GABA	His	Total
Untransformed tomato											
Control-1	1.31	0.10	0.14	0.26	8.88	0.68	0.02	0.30	0.45	0.13	12.77
Control-2	1.04	0.07	0.14	0.21	8.48	0.39	0.02	0.22	0.45	0.13	11.90
Plasmid (pMAT037) alone											
pMAT-1	1.33	0.14	0.32	0.23	6.00	0.70	0.04	0.66	0.14	0.17	10.31
pMAT-2	2.00	0.20	0.37	0.23	11.18	0.50	0.06	0.83	0.62	0.25	17.09
pMAT-3	1.28	0.22	0.44	0.37	3.27	0.85	0.14	0.13	0.22	0.25	12.22
***AN-gdh-17*gene introduced transformant**											
No. 6	1.37	0.29	0.70	0.42	15.16	0.65	0.15	1.59	0.46	0.39	22.86
No. 8-2	2.66	0.18	0.51	0.27	13.97	0.65	0.07	1.19	0.27	0.32	20.82
No. 15	5.51	0.43	0.94	1.18	22.08	4.29	0.16	2.53	1.98	0.53	41.42
No. 17	3.19	0.24	0.69	0.38	21.55	0.67	0.12	1.75	0.82	0.37	30.70

(μmol/g F.W.)

The amino acid analysis of the 4 strains containing tomato T-gdh-4 gene introduced therein was conducted by using the fruits taken 6 weeks after the blooming (Table 5). In the plant lines wherein the remarkable increase in glutamic acid content was observed, glutamic acid content was increased to 2.28 times (No. 2), 3.52 times (No. 7-2), 2.74 times (No. 9-2) and 2.53 times (No. 10) (Fig. 15). In the plant lines of a high glutamic acid content, amino acids other than glutamic acid, such as aspartic acid, asparagine, threonine, serine, alanine and histidine were also increased in amount. The total amino acid content was increased to 4 times (No. 7-2). The results are summarized in Table 5.

Table 5. Amino acid content of transformed tomato (T_0 generation) into which *T-gdh-4* gene was introduced

	Asp	Thr	Ser	Asn	Glu	Gln	Gly	Ala	GABA	His	Total
Untransformed tomato											
Control-1	1.31	0.10	0.14	0.26	8.88	0.68	0.02	0.30	0.45	0.13	12.77
Control-2	1.04	0.07	0.14	0.21	8.48	0.39	0.02	0.22	0.45	0.13	11.90
Plasmid (pIG121-Hm) alone											
pIG-1	2.13	0.20	0.37	0.36	9.40	1.07	0.07	1.02	0.33	0.26	16.08
pIG-2	1.03	0.12	0.27	0.16	6.59	0.30	0.07	0.68	0.23	0.15	10.10
pIG-3	0.67	0.07	0.21	0.20	3.64	0.15	0.03	0.35	0.14	0.26	5.89
***T-gdh-4* gene introduced transformant**											
No. 2	4.01	0.27	0.49	0.64	19.76	0.75	0.08	1.09	0.59	0.37	28.93
No. 7-2	6.21	0.55	0.56	0.91	30.56	4.71	0.08	0.80	3.20	0.74	50.15
No. 9-2	4.27	0.69	1.15	1.14	23.81	3.97	0.23	2.25	1.04	0.70	42.17
No. 10	6.78	0.37	0.87	0.62	21.96	1.21	0.15	1.83	2.41	0.63	38.03

(μmol/g F.W.)

Example 3. Analysis of subsequent generation (T_1) of tomato transformant

(1) Selection of T_1 generation

Seeds of transformed tomato (T_0 generation) obtained in Example 2 and several lines of the transformants (T_0 generation) obtained by similar procedure were surface-sterilized with 80 % ethanol for 30 seconds and 2 % sodium hypochlorite for 15 minutes, and then planted in MS agar medium containing 350 mg/l of kanamycin under sterile conditions. One month later, well-grown plants were selected to obtain the selected plant bodies from No.1, 3, 15 and 2.1 in *AN-gdh*-17 transgenic lines and from No.1, 3, and 8 in *T-gdh*-4 transgenic lines. The plants were cultured in an outdoor closed system greenhouse in order to increase the number of fruits per plant. To make the nutrition conditions uniform, no additional fertilizer was given after the

transplantation into 1 kg of culture soil (Power soil; *Sakata no Tane*) during the acclimation to the soil. In the following analysis, leaves were not picked in order to uniform the assimilation, and the lateral buds were cultivated under uniform conditions. The leaf tissues of the thus obtained were used.

(2) Confirmation of the introduced gene by Southern analysis

Total DNA was extracted from leaf tissues of lateral buds of the acclimated plant [Honda and Hirai, Jpn. J. Breed 40, 339-348 (1990)]. 15 μg of DNA was treated with the combination of restriction enzymes *Bam*HI and *Eco*RI and also reacted with *Xba*I. After electrophoresis, it was transferred onto a nylon membrane. The obtained product was then subjected to Southern hybridization with a DIG-Labeling and Detection Kit (Roche Molecular Biochemicals) using *AN-gdh-17* gene or *T-gdh-4* gene as the probes.

Southern hybridization was conducted with *AN-gdh-17* gene as the probe. As a result, bands of intended size (1.8 kb, 0.8 kb) were observed in No. 1, No. 3, No. 15 and No. 2.1 in AN-gdh-17 gene transgenic lines and the introduction of the gene was confirmed (Fig. 16). In the same manner, Southern hybridization was conducted with *T-gdh*-4 gene as the probe. A band (1.2 kbp) of a size equal to that of *T-gdh*-4 gene was confirmed. In several plants, a band was observed around the location of 20kbp, which was considered as the endogenous GDH gene (Fig. 17).

3) Determination of activity of NADP-GDH and NAD-GDH

Leaf tissue (0.2 g) of lateral buds of transformed tomato (T_1) was frozen in liquid nitrogen, and then crushed in a mortar. 5-fold weight of an extract buffer [200 mM Tris (pH 8.0), 14 mM β-mercaptoethanol, 10 mM L-cysteine-HCl, 0.5 mM PMSF, 0.5 % Triton X-100] was added. The obtained mixture was transferred into a centrifugal tube and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was ultrafiltrated (Millipore, ultrafree 0.5 filter unit, Biomax-10) and washed with the extraction buffer three times.

The extracted enzyme was mixed with a reaction mixture [100 mM Tris (pH 8.0), 20 mM 2-α-ketoglutarate, 1.0 mM $CaCl_2$, 0.2 mM NADPH (for NADP-GDH activity determination) or 0.2 mM NADH (for NAD-GDH activity determination), 200 mM ammonium chloride], and the reaction was carried out at room temperature. The reduction in the absorbance at 340 nm was determined.

NADP-GDH activity was determined by using leaf tissue of transformed tomato (T_1) containing *AN-gdh*-17 gene introduced therein. The activity of the transformant could be determined to be 230 to 400 nmol/(min.mg protein), while no activity of the untransformed product was recognized (Table 6). NAD-GDH activity of the line containing *T-gdh-4* gene introduced therein was increased 2-fold or more compared to that of the non-transformant (Table 7).

Table 6. NADP-GDH activity of transformed tomato with *AN-gdh-17* gene.

Lines	Activity of NADP-GDH (nmol/(min.mg protein))
Untransformed tomato	0
Transformed tomato	
AN-gdh-17 No. 1-1	400
AN-gdh-17 No. 3-1	390
AN-gdh-17 No. 15-1	380
AN-gdh-17 No. 2.1-1	230

Table 7.NAD-GDH activity of transformed tomato with *T-gdh-4* gene

Lines	Activity of NAD-GDH (nmol/(min.mg protein))
Untransformed tomato	80
Transformed tomato	
T-gdh No. 1-2	180
T-gdh No. 3-1	160
T-gdh No. 8-1	260

4) Determination of amino acid content in fruits

Three fruits taken in the 6th week after blossoming of the first fruit cluster were used for the analysis. 3 parts by weight of 80 % ethanol heated to 80°C was added to 1 part by weight of the fruits. The obtained mixture was ground in a mortar and then heated again to 80°C for 20 minutes. After centrifugation at 7,000 rpm, the obtained supernatant was recovered. After the addition of 80 % ethanol, the obtained mixture was heated to 80°C. The ethanol extraction was conducted three times, and the obtained extracts were combined together and then 80 % ethanol was added thereto to make the total amount 100 ml. After thoroughly mixing, 200 μl of the extract was taken in an Eppendrof tube, dried and then dissolved in 200 μl of sterilized water. 200 μl of ethyl ether was added to the obtained solution, and they were mixed together and then centrifuged at 12,000 rpm. The ether layer was removed. The aqueous layer was dried again and dissolved in 200 μl of 0.02 N HCl. The resultant solution was filtered through a 0.45 μm filter, and the filtrate was taken as a sample and analyzed with Hitachi high-speed amino acid analyzer (L-8800).

The results were shown by the average of three fruits. Glutamic acid contents of AN1-1-2 and AN1-1-3 from *AN-gdh-17* No. 1 line were increased to

2.1 times and 2.8 times as high as that of the untransformed fruit, respectively. Also, glutamic acid contents of AN3-1-2 and AN3-1-3 from *AN-gdh-17* No. 3 line were increased to 2.8 times and 2.5 times as high as that of the untransformed fruit. Further, glutamic acid contents of AN15-1 from *AN-gdh-17* No. 15 line and AN2.1-1-1 from *AN-gdh-17* No. 2.1 line were also increased to 2.1 times and 1.9 times, respectively (Table 8, Fig. 18). The similar tendency was observed also in the subsequent generation of No. 15 line whose glutamic acid content was high in the transformed generation (T_0).

Table 8. Amino acid contents in fruits of the progenies (T_1) of *AN-gdh-17* gene introduced tomato transformants

	Asp	Thr	Ser	Asn	Glu
Control	1.09±0.48	0.10±0.03	0.22±0.05	0.18±0.09	6.42±1.16
AN-GDH 1-1-2	2.50±0.78	0.19±0.02	0.41±0.02	0.38±0.04	13.71±2.55
AN-GDH 1-1-3	4.17±0.15	0.42±0.06	0.86±0.09	0.63±0.13	18.42±0.99
AN-GDH 3-1-2	4.18±0.96	0.30±0.07	0.66±0.19	0.50±0.18	18.39±2.74
AN-GDH 3-1-3	3.37±0.89	0.33±0.05	0.71±0.12	0.57±0.07	16.25±0.73
AN-GDH 15-1	2.18±0.16	0.15±0.02	0.36±0.02	0.29±0.01	13.95±0.28
AN-GDH 2.1-1-1	2.33±0.38	0.20±0.04	0.46±0.17	0.23±0.06	12.43±0.77

Table 8. (Continued)

	Gln	Ala	His	GABA	Total
Control	0.44±0.33	0.29±0.11	0.13±0.04	0.96±0.11	10.36±4.24
AN-GDH 1-1-2	0.45±0.11	0.62±0.16	0.23±0.02	2.86±0.58	22.18±3.71
AN-GDH 1-1-3	1.50±1.20	1.54±0.15	0.44±0.02	5.67±0.18	35.46±2.88
AN-GDH 3-1-2	1.05±0.91	1.25±0.84	0.39±0.08	3.33±0.97	31.76±4.76
AN-GDH 3-1-3	1.49±1.02	1.18±0.48	0.39±0.06	2.90±0.40	28.68±1.22
AN-GDH 15-1	0.27±0.10	0.73±0.13	0.23±0.10	1.09±0.13	19.97±0.25
AN-GDH 2.1-1-1	0.19±0.03	0.91±0.47	0.24±0.10	1.84±0.09	19.59±2.29

(μmol/g.F.W.) (n=3)

In *T-gdh-4* transgenic lines, glutamic acid content of T-gdh1-2, 3-1 and

8-1, which were the subsequent generations of examined *T-gdh-4*, Nos. 1, 3 and 8 lines, were increased to 2.3 times, 2.1 times and 2.4 times, respectively, as high as that of the untransformed fruit (Table 9, Fig. 19). As for amino acids other than glutamic acid, a remarkable increase in aspartic acid content, glutamine content and γ-amino butyric acid content was observed. As a result, the total free amino acid content was also increased 2- to 3-fold as compared to the untransformed plant.

Table 9. Amino acids contents in fruits of the progenies (T_1) of *T-gdh-4* gene introduced tomato transformants

	Asp	Thr	Ser	Asn	Glu
Control	1.09±0.48	0.10±0.03	0.22±0.05	0.18±0.09	6.42±1.16
T-gdh 1-2	2.61±0.51	0.28±0.05	0.56±0.12	0.48±0.02	14.74±3.12
T-gdh 3-1	2.41±0.51	0.20±0.04	0.44±0.13	0.30±0.01	13.81±2.64
T-gdh 8-1	2.85±0.60	0.22±0.05	0.46±0.11	0.31±0.07	15.70±3.47
	Gln	Ala	His	GABA	Total
Control	0.44±0.33	0.29±0.11	0.13±0.04	0.96±0.11	10.36±4.24
T-gdh 1-2	0.79±0.65	0.91±0.56	0.29±0.17	1.31±0.96	23.29±3.88
T-gdh 3-1	0.43±0.37	0.70±0.35	0.27±0.06	1.53±0.48	21.09±2.80
T-gdh 8-1	0.46±0.07	0.69±0.05	0.23±0.18	2.24±0.85	24.22±5.19

(μmol/g. F.W.) (n=3)

Example 4. Production and analysis of potato transformants

(1) Production of transformants

Sterile potatoes were obtained by shoot apex culture. The materials

were propagated by shoot apex subculture. The shoot apexes was placed in a liquid culture medium (10 ml) prepared by adding 2 % sucrose to MS medium to induce the rooting. After completion of the rooting, 10 ml of MS liquid medium containing 16 % sucrose was added to the medium, and dark culture was conducted to induce the formation of microtubers. The 6 to 8 week old microtubers were cut to form disc-shaped pieces. After peeling, the pieces were infected with an *Agrobacterium* suspension (Ti-plasmid. pMt-dAN-gdh or pCt-AN-gdh) cultured at 28°C overnight. A sterilized filter paper was placed on MS agar medium (MS medium, 2.0 mg/l Zeatin, 0.1 mg/l indole acetic acid, 0.3 % gelrite), the pieces were placed thereon and co-cultured at 25°C for 2 days while the daylight hours were kept to be 16 hours. The culture was then transferred to a selection medium (MS medium, 2.0 mg/l Zeatin, 0.1 ml/l indole acetic acid, 0.3 % gelrite, 50 mg/l kanamycin and 500 mg/l claforan) and cultured under the same conditions as those described above. The discs were transferred into the fresh screening medium every week, and the differentiated shoots were transferred into a plant hormone-free selection medium to induce the rooting. After infection with Agrobacterium harboring Ti-plasmid pMt-dAN-gdh or pCt-AN-gdh and selection on the medium containing 50mg/l kanamycin, 4 lines, Mt-dAN-gdh No.2, 5, 8 and Ct-AN-gdh No.1, were obtained.

2) Confirmation of the introduced gene by Southern analysis

Total DNA was extracted from leaf tissues of the acclimated plant [Honda and Hirai, Jpn. J. Breed 40, 339-348 (1990)]. 15 μg of DNA was treated with restriction enzyme *Eco*RI. After electrophoresis, it was transferred on a nylon membrane. Southern hybridization was carried out using DIG-Labeling and Detection Kit (Roche Molecular Biochemicals). *AN-gdh-17* gene was used as the probe.

As a result, the band of the intended size (about 1.5kb) was confirmed in all 4 lines (Fig. 20), which suggested that *gdh* gene connected to the transit peptide was introduced therein.

3) Determination of activity of NADP-GDH

Leaf tissues (about 0.1 g) of transformed tomato were frozen in liquid nitrogen, and then crushed in a mortar. 5-fold weight of the extract buffer [200 mM Tris (pH 8.0), 14 mM β-mercaptoethanol, 10 mM L-cysteine-HCl, 0.5 mM PMSF, 0.5 % Triton X-100] was added. The obtained mixture was transferred into a centrifugal tube and centrifuged at 12,000 rpm for 10 minutes. The supernatant was ultrafiltrated (Millipore, ultrafree 0.5 filter unit, Biomax-10) and washed with the extract buffer three times. The extracted enzyme was mixed with a reaction solution [100 mM Tris (pH 8.0), 20 mM 2-α-ketoglutarate, 1.0 mM $CaCl_2$, 0.2 mM NADPH, 200 mM ammonium chloride], and the reaction was carried out at room temperature. The reduction in the absorbance at 340 nm was determined.

NADP-GDH activity was determined by using the leaf tissues of the transformed potatoes in which the introduced gene could be confirmed by Southern analysis, and the untransformed potatoes. As a result, the activity of the transformants could be determined to be 150 to 300 nmol/(min.mg protein), while no activity of the untransformed product was recognized (Table 10). Mt-dAN-gdh lines showed higher activity than Ct-AN-gdh lines.

Table 10. NADP-GDH activity of transgenic potato in which *Mt-dAN-gdh* or *Ct-AN-gdh* gene was introduced

Line	Activity of NADP-GDH (nmol/(min.mg protein))
Untransformed potato	0
Transformed potato	
Mt-dAN-gdh No. 2	290
Mt-dAN-gdh No. 5	300
Mt-dAN-gdh No. 8	260
Ct-AN-gdh No. 1	150

4) Determination of amino acid content in microtubers

The shoot apexes of 4 transformant lines and non-transformant line were liquid-cultured to induce the rooting, and then 16 % sucrose was added to the culture medium. 6 weeks after the dark-treatment, amino acid content of the microtubers was determined.

3 parts by weight of 80 % ethanol heated to 80°C was added to 1 part by weight of the microtubers. The obtained mixture was ground in a mortar and then heated again to 80°C for 20 minutes. After the centrifugation at 7,000 rpm and the obtained supernatant was recovered. After the addition of 80 % ethanol, the obtained mixture was heated to 80℃. The ethanol extraction was conducted three times, and the obtained extracts were combined together and then 80 % ethanol was added thereto to adjust the total amount to 5 ml. After thoroughly mixing, 200 μl of the extract was taken in an Eppendrof tube, dried and then dissolved in 200 μl of sterilized water. 200 μl of ethyl ether was added to the obtained solution, and they were mixed together and then centrifuged at 12,000 rpm. The ether layer was removed. The aqueous layer was dried again and dissolved in 400 μl of 0.02 N HCl, and the solution was filtered through a 0.45 μm filter, and the filtrate was taken as a sample and analyzed with Hitachi high-speed amino acid analyzer (L-8800).

Amino acid analysis of microtubers derived from the transgenic lines was conducted. At least two microtubers were analyzed for each line, and the analytical results were statistically treated. Glutamic acid contents of Mt2-2, Mt5-1, Mt5-2, Mt8-1 and Mt8-2 lines from No 2, 5 and 8 plant lines into which *Mt-dAN-gdh* gene had been introduced were increased to 1.7 times, 2.2 times, 2.5 times, 3.0 times and 2.2 times as high as that of the untransformed sample, respectively (Table 11, Fig. 21). In the plant lines into which *Ct-AN-gdh* gene had been introduced, no significant difference in glutamic acid content from the untransformed sample was recognized. As for amino acids other than glutamic acid, a remarkable increase in glutamine content and proline content was observed. Consequently, the total free amino acid content was also increased to 2 to 3 times as high as that of the untransformed plant.

Table 11. Amino acid contents in microtubers of potatoes into which *Ct-AN-gdh* or *Mt-dAN-gdh* gene was introduced

	Asp	Thr	Ser	Asn
Control	1.08±0.23	0.48±0.13	0.66±0.25	13.77±6.24
CtAN-gdh no.1-1	1.47±0.36	0.79±0.22	1.57±0.21	42.12±17.05
MtdAN-gdh no.2-2	1.85±0.01	0.88±0.02	1.31±0.03	14.79±2.40
MtdAN-gdh no.5-1	1.62±0.03	0.84±0.29	1.32±0.54	11.12±2.55
MtdAN-gdh no.5-2	4.17±3.01	0.74±0.09	1.44±0.02	28.62±3.28
MtdAN-gdh no.8-1	2.32±0.61	0.88±0.19	1.57±0.24	30.97±2.16
MtdAN-gdh no.8-2	2.00±0.28	0.83±0.15	1.12±0.13	16.69±2.80
	Glu	Gln	Ala	His
Control	2.25±0.58	10.00±4.25	0.52±0.30	0.25±0.08
CtAN-gdh no. 1-1	3.16±1.40	17.74±5.29	1.42±0.59	0.80±0.43
MtdAN-gdh no.2-2	3.75±0.03	27.48±1.98	1.67±0.03	0.26±0.04
MtdAN-gdh no.5-1	4.98±1.93	34.09±8.11	1.14±0.51	0.59±0.30
MtdAN-gdh no.5-2	5.54±0.95	25.31±3.20	1.86±0.17	0.36±0.12
MtdAN-gdh no.8-1	6.71±2.28	20.07±4.90	1.89±0.25	0.46±0.28
MtdAN-gdh no.8-2	4.86±1.14	14.56±2.93	1.06±0.19	0.28±0.13
	GABA	Arg	Pro	Total
Control	1.29±0.53	0.46±0.16	5.03±4.21	38.57±10.31
CtAN-gdh no. 1-1	2.10±1.19	2.93±1.61	15.32±9.30	93.48±20.00
MtdAN-gdh no.2-2	3.22±0.52	0.78±0.11	10.55±1.73	71.66±1.84
MtdAN-gdh no.5-1	2.96±1.74	2.00±1.24	8.65±5.24	73.45±22.78
MtdAN-gdh no.5-2	1.10±0.21	2.18±1.27	11.58±0.73	86.12±3.35
MtdAN-gdh no.8-1	2.03±1.15	1.53±0.81	25.03±1.06	98.66±26.85
MtdAN-gdh no.8-2	1.61±0.47	0.68±0.09	7.71±2.09	55.40±6.08

(μmol/g. F.W.) ($n \geq 2$)

Example 5. Investigation on the yield of potato transformants

The yield of several *Mt-dAN-gdh* transgenic potato lines obtained in Example 4 and the lines obtained by similar method were tested.

The sterile transformed plants were acclimatized, and then transferred

to pots (Type 7) containing 2.5kg of power-soil (Table 12). They were cultivated under natural light at 25°C for 2 months (in a closed system greenhouse, from March to May, 2001) providing only with water and without top-dressing. At the end of the period of cultivation, the above-ground parts and the tuber parts were weighed, and the number of tubers and stems were also measured. The results were shown in Table 13 and Figs.22 and 23.

Table 12. Composition of power-soil

Item	Contents
Water	14%
Distribution of particles	Diameter of particle 0.5-3mm
pH	about 6.5
Amount of added fertilizer (per 1kg)	Total nitrogen 0.40g nitrate-nitrogen 0.05g ammonia-nitrogen 0.35g Total phosphate 2.00g Water soluble potassium 0.60g Hardly soluble magnesia 0.20g

Table 13. Yield of potato transformant

	Fresh weight of above-ground parts (g)	Total weight of tuber parts(g)	Number of tubers	Number of stems
Non-transformant no.1	5.8	53.9	9	1
Non-transformant no.2	8.2	54.7	7	1
Transformant Mt-dAN no.1-1	56.4	175.6	14	2
Transformant Mt-dAN no.2-3	26.4	89.0	10	2
Transformant Mt-dAN no.3-1	53.8	228.9	15	3
Transformant Mt-dAN no.5-3	41.6	118.0	19	2
Transformant Mt-dAN no.8-3	64.4	224.2	16	3

A remarkable increase in the number of tubers and in the total weight of tuber parts was observed in *Mt-dAN-gdh* gene introduced potatoes.

According to the present invention, plants containing free amino acids in a high concentration can be obtained. Thus, crops usable as starting materials and food materials having a high added-value are provided. According to the present invention, the whole free amino acid content is increased 2 to 4-fold. Particularly crops containing a very high concentration of at least one of glutamic acid, asparagine, aspartic acid, serine, threonine, alanine and histidine are provided. Thus, crops to be used as starting materials having a high added-value, which do not require the addition of these amino acids, are provided. Further, according to the present invention, vegetables which can be directly cooked and which contains a high concentration of glutamic acid accumulated therein, namely food materials having a good taste, can be provided.

In addition, the period for breeding plants containing such free amino acids in a high concentration is remarkably shortened according to the present invention.

Furthermore, the yield of potato can be increased according to the present invention. The total weight of tuber parts of potato increases at least about 1.5-fold and the number of tubers per plant body significantly increases.